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ANALYSIS OF MUTATIONS AND IDENTIFICATION OF SEVERAL POLYMORPHISMS IN THE PUTATIVE PROMOTER REGION OF THE P34[CDC2]-RELATED *CDC2L1* GENE LOCATED AT 1P36 IN MELANOMA CELL LINES AND MELANOMA FAMILIES

Yongmei Feng[1,2], Jiaqi Shi[1,2], Alisa M. Goldstein[3], Margaret A. Tucker[3] and Mark A. Nelson[1,2]*

[1]*Arizona Cancer Center, Tucson, AZ, USA*
[2]*Pathology Department, University of Arizona, Tucson, AZ, USA*
[3]*Genetic Epidemiology Branch, National Cancer Institute, Bethesda, MD, USA*

Chromosome 1 abnormalities are the most commonly detected aberrations in many cancers including malignant melanoma. Partial deletions and an allelic loss of the chromosome 1p36 region observed in melanoma indicate the presence of putative tumor suppressor gene(s) in this region. A candidate gene, *CDC2L1*, which encodes PITSLRE proteins related to p34[cdc2], is mapped to 1p36. To determine whether *CDC2L1* mutation is involved in melanoma development, we examined 20 melanoma cell lines and 11 members of melanoma-prone families linked to chromosome 1p36. Mutation analysis throughout the entire coding region of the *CDC2L1* gene revealed only 1 mutation (C→T at nucleotide location 97 of exon 7, Ser→Leu) in the melanoma cell line UACC 903 out of 20 melanoma cell lines and 6 melanoma cases. However, 4 polymorphic nucleotide changes, C-48T, G-53C, T-103C and T-210C, in the putative promoter region of *CDC2L1* were identified. The 4 variants were located within or beside the conserved binding sites of transcription factors TCF11, MZF1 and TAAC box, indicating their potential effects on the regulation of *CDC2L1* expression. No aberrant methylation of the *CDC2L1* CpG island in the promoter region was observed by sodium bisulfite genomic sequencing. These results indicate that mutations are rare in the *CDC2L1* gene in these melanoma cell lines and melanoma families and that the aberrant cytosine methylation of the *CDC2L1* CpG island is not the mechanism of *CDC2L1* repression in melanoma. The contribution of 4 promoter polymorphisms to the transcriptional regulation of the gene and its association with melanoma warrants further investigation.
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The incidence of melanoma in the United States is increasing faster than that of any other human cancers. Although a few genetic alterations have been identified in melanoma, relatively little is known regarding the molecular mechanism that underlies the etiology of this disease. The identification of associated genetic changes would help to advance the understanding of the development of melanoma and could lead to new and innovative strategies to treat this disease.

PITSLRE protein kinases are part of the large family of p34[cdc2]-related kinases whose functions appear to be linked to the control of cell division and possibly programmed cell death.[1–4] Several lines of evidence suggest that 1 or more PITSLRE kinase isoforms may be tumor suppressor genes.[4–6] For example, low levels of PITSLRE mRNA and protein levels in CHO cells lead to enhanced cell growth, whereas increased levels of PITSLRE mRNA and protein lead to decreased cell growth.[4] Recent studies by our group demonstrated that activation of PITSLRE protein is a downstream event in Fas-induced apoptosis in melanoma.[7] In addition, decreased PITSLRE expression and altered localization may result in the loss of apoptotic signaling.[7] Thus, 1 or more of the PITSLRE kinase isoforms may be involved in apoptotic signal transduction.

Chromosome band region 1p36 is frequently deleted in a variety of human tumors such as malignant melanoma, neuroblastoma, colon cancer and breast cancer.[8] Presumably these deletions represent loss of 1 or more tumor suppressor genes. The *CDC2L* locus encoding PITSLRE protein kinases maps to chromosome 1p36.3[9] and consists of 2 duplicated and tandemly linked genes *CDC2L1* and *CDC2L2*.[10] The 2 genes are almost identical, yet transcripts are expressed ubiquitously from these genes in most human cell lines and tissues examined. Two CpG-rich sequences upstream of the gene are responsible for the expression of transcripts from *CDC2L1* and *CDC2L2*.[10]

In our previous study, we found that 1 allele of the *CDC2L1* gene complex on chromosome 1 was either deleted or translocated in 8 of 14 different melanoma cell lines using fluorescence *in situ* hybridization[11] and that the alterations in these alleles correlate with differences in sensitivity of melanoma cells to apoptotic stimuli. Decreased expression of PITSLRE proteins from the remaining allele was observed in several cell lines and surgical malignant melanoma specimens.[11] Similar alterations in the *CDC2L1* locus were also observed on chromosome 1p36 in childhood endodermal sinus tumors,[12] neuroblastoma cell lines[13] and lymphomas.[14] The purpose of our study was to screen *CDC2L1* gene variants in melanoma cell lines and melanoma families and to investigate the possible role of the gene in melanoma development and progression.

MATERIAL AND METHODS

Melanoma cell lines and tumor specimens

Human melanoma cell lines were obtained from the Arizona Cancer Center Tissue Culture Shared Resource. The cells were grown as monolayers in RPMI-1640 containing 5% dialyzed and heat-inactivated FCS, 1% L-glutamine and 1% penicillin-streptomycin. Normal human epidermal melanocytes were purchased from Cascade Biologics (Portland, OR) and cultured in M154 medium containing human melanocyte growth supplement (HMGS) and 1% penicillin-streptomycin following the manufacturer's instruction. Other normal cell lines included normal fibroblasts, peripheral blood lymphocytes (PBLs) and normal lung (NSL1). Melanoma family DNA samples (derived from PBLs) used in our study were obtained from the NCI (NCI family numbers R, J and AH) and exhibited linkage to chromosome 1p36.[15–17]

DNA preparation

Genomic DNA was extracted from the cell lines as described below. Briefly, 5×10^5 cells were harvested from the culture and

*Correspondence to: Arizona Cancer Center, 1515N Campbell Ave. Tucson, AZ 85724, USA. Fax: +520-626-8447.
E-mail: mnelson@azcc.arizona.edu

washed twice with PBS before incubation for 3 hr at 55°C in 1 ml of 10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 150 mM NaCl, 0.5% SDS and 200 μg/ml proteinase K. Phenol, phenol/chloroform (1:1) and chloroform extractions were then performed before DNA precipitation with sodium acetate and ethanol. For these samples, 100 ng of DNA was used for each PCR reaction. Quality control of the DNA and quantification of the amount of DNA was done by electrophoresis and spectrophotometry.

PCR amplification

PCR primer pairs were designed to cover the entire 20 exons and correspond to the flanking intron sequences of the *CDC2L1* gene. Primers used to amplify all the exons and 5′UTR of the PITSLRE gene are summarized in Table I. PCR reactions (50 μl) were carried out using 100 ng genomic DNA, 0.25 mM dNTPs, 1 μM of each primer and 1 unit of *Taq* DNA polymerase (Takara, Fisher, Fair Lawn, NJ) in the reaction buffer provided by the supplier. Thermal cycling consisted of an initial denaturation at 94°C for 5 min followed by 30–40 cycles of denaturing at 94°C (30 sec), annealing at the temperature specific for each pair of primers (30 sec) and extension at 72°C (60 sec), with a final 10 min extension at 72°C. PCR fragments were isolated by gel electrophoresis and purified prior to sequencing using the GFX PCR purification kit (Pharmacia Biotech, Gaithersburg, MD).

Restriction enzyme analysis

Sequence analysis of the 5′ upstream sequence revealed a C→T change destroying a *Xho*I restriction site. This site was used to confirm the presence of the changes in melanoma cell lines and melanoma family members. A 309 bp region of the *CDC2L1* 5′ upstream sequence was PCR-amplified from genomic DNA. PCR products were column-purified (Pharmacia Biotech) and digested with *Xho*I (Gibco, Grand Island, NY) at 37°C for 2 hr. Digested DNAs were subjected to gel electrophoresis on a 2% agarose gel. Three possible genotypes are defined by 3 distinct banding patterns: T/T (309 bp fragment), C/T (309 bp, 154 bp and 155 bp fragments) and C/C (154 bp and 155 bp fragments). Restriction digestions were carried out in 20 μl reaction. PCR product (10 μl) was digested using 2 units of restriction enzyme at 37°C for 2 hr.

Bisulfite modification and bisulfite genomic sequencing

Bisulfite modification of genomic DNA was performed as reported by Rice *et al.*[18] Briefly, 5 μg of genomic DNA was denatured with NaOH (final concentration, 0.3 M), and 10 mM hydroquinone (Sigma, St. Louis, MO) and 3.6 M sodium-bisulfite (Sigma) were added and incubated at 55°C for 14 hr. Afterward, modified DNA was desalted using a Wizard Prep kit (Promega, Madison, WI) and treated with 0.3 M NaOH followed by ethanol precipitation. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines (but not methylated cytosines) to uracil, which are then converted to thymidine during the subsequent PCR step, giving sequence differences between methylated and unmethylated DNA. The *CDC2L1* promoter CpG island was amplified from the bisulfite-modified DNA by 2 rounds of PCR using nested primers specific to the bisulfite-modified sequence of the *CDC2L1* CpG island.

Primers used to amplify the modified DNA are described in Table I. The primers were designed so that the original CpG dinucleotides were avoided. Primers 1 and 2 were used in the first round of amplification under the following conditions: 95°C for 1 min followed by 35 cycles of 92°C for 1 min, 56°C for 3 min and 72°C for 1 min, ending with a final extension of 72°C for 5 min. One to 10% of the first-round PCR product was used for the second round of PCR using primers 3 and 4 under the same conditions. The resultant PCR product was cloned into a TA vector according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). Positive recombinants were isolated using a QIAprep Spin Miniprep Kit (Qiagen, Chatsworth) according to the manufacturer's instructions and sequenced on an ABI automated DNA sequencer. The methylation status of individual CpG sites was determined by comparison of the sequence obtained with the known *CDC2L1* sequence.

DNA sequencing

The same primers used for PCR amplification were used for direct DNA sequencing, after purification of the samples by GFX columns. Sequencing was performed by the DNA Sequencing Service at the University of Arizona, by the dideoxy termination method and an ABI 377 automated sequencer. Nucleotide differences were determined by comparison with the sequences deposited in GenBank. Results were verified by sequencing both the sense and the antisense strands. A sample was considered to have a mutation only if the nucleotide change was present at the same position on both strands.

RESULTS

Detection of CDC2L1 gene mutations in melanoma cell lines

Only 1 mutation was detected in 20 melanoma cell lines tested. Specifically, the melanoma cell line UACC903 contains a mutant *CDC2L1* allele. A transition from cytosine to thymine at nucleotide location 97 in exon 7 of the *CDC2L1* gene was observed. This alteration occurs in the second nucleotide of the codon and yields an amino acid alteration of Ser→Leu (amino acid position 233). Normal melanocytes, fibroblasts, PBLs and NSL1 were used as wild-type controls. In addition, some minor differences were observed between our *CDC2L1* sequence and the sequence listed in GenBank (accession numbers AF080687 and AF080688). These included C rather than T at nucleotide 63 in exon 4 and 2 G rather than A at nucleotides 53 and 110 in exon 5.

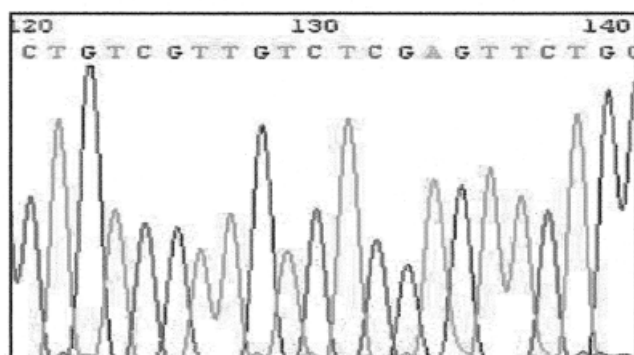### Detection of CDC2L1 gene mutations in melanoma families

We also analyzed DNA samples of 6 individuals from 3 melanoma-prone kindreds putatively linked to 1p36 and 5 samples from control individuals (3 unaffected family members and 2 spouses). All 20 exons of the *CDC2L1* gene were amplified and subjected to direct sequencing for detecting nucleotide variants of *CDC2L1* gene in these families. Mutation analysis of these 11 samples (6 cases, 5 controls) did not identify any distinct mutations in the *CDC2L1* gene.

Polymorphisms in the 5′ putative promoter region of CDC2L1 gene
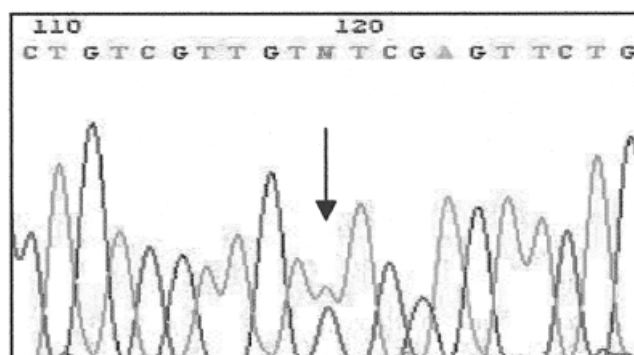
A total of 4 common polymorphisms were identified within the putative promoter region of the *CDC2L1* gene in melanoma cell lines and melanoma families (Table II). Change 1 was C→T substitution at nucleotide −48. Change 2 was G→C substitution at −53. Changes 3 and 4 were T→C substitutions at nucleotide positions −103 and −210, respectively. A representative chromatogram of DNA sequencing of polymorphism change C-48T is shown in Figure 1. For our report, numbering of the putative *CDC2L1* promoter is relative to the first nucleotide of exon 1, so that the first nucleotide of exon 1 is numbered +1, and the nucleotide immediately preceding is numbered −1.

For polymorphism changes C-48T, T-103C and T-210C, even genotype distribution was observed in melanoma cases (2 patients over a total of 6 patients tested against each A1/A1 wild type, A1/A2 heterozygotes and A2/A2 homozygotes; Table II). However, although our results are based on small numbers, there was no evidence for segregation of the variants with disease across the 3 families (data not shown). Change G-53C appeared to be a rarer polymorphism because only 1 heterozygous G/C was found in unaffected members of melanoma families, and no homozygous C/C was found in all the melanoma family samples tested. For the melanoma cell lines, changes C-48T, T-103C and T-210C expressed the same genotype distribution, 14/20 for homozygous A1/A1 wild type, 5/20 for heterozygous A1/A2 and 1/20 for homozygous A2/A2. No homozygous A2/A2 genotype was detected in cell line control groups for any of the 4 changes. Change
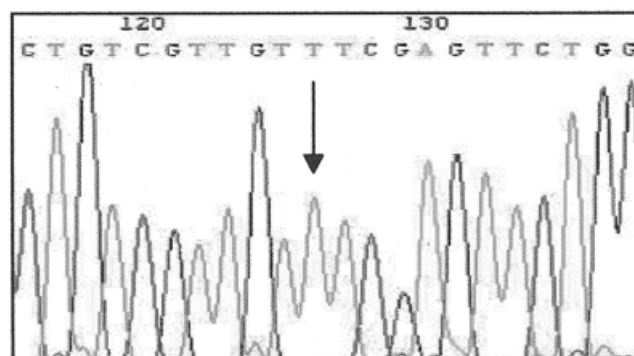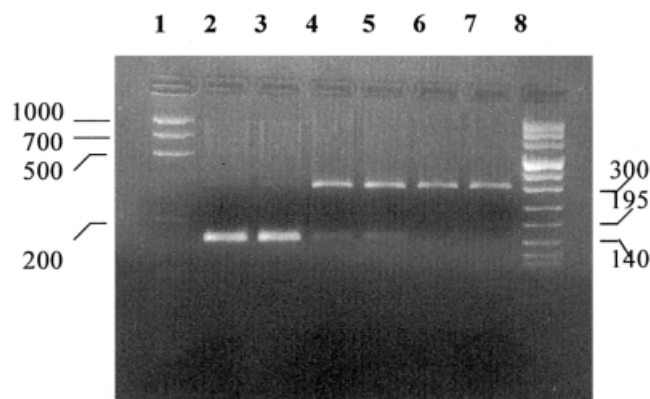
A. Normal melanocytes

B. A375

C. UACC 2972

FIGURE 1 – Representative chromatograms of DNA sequencing of the polymorphism change C-48T in the putative promoter region of the *CDC2L1* gene. (*a*) Normal melanocytes; (*b*) A375 and (*c*) UACC 2972. Arrows indicate the base change of C→T in A375 (1 allele) and UACC 2972 (2 alleles).

FIGURE 2 – Representative PCR-restriction length analysis of the polymorphism change C-48T in the putative promoter region of the *CDC2L1* gene after digestion with *Xho*I. Homozygous wild type C/C-derived amplicons *Xho*I digestion resulted in 154 bp and 155 bp fragments, homozygous T/T-derived amplicons *Xho*I digestion remained uncut (309 bp), while heterozygous T/C resulted in half uncut (309 bp) and half cut (154 bp and 155 bp) fragments. Lanes 1 and 8, DNA ladder; lanes 2 and 3, homozygous wild type C/C; lanes 4 and 5, heterozygous T/C; lanes 6 and 7, homozygous T/T.

CDC2L1 promoter PCR fragment, producing bands of 154 bp and 155 bp if the mutant T-allele was present at −48, but not if the wild-type C-allele was present. The 3 possible genotypes are defined by 3 distinct banding patterns.

By using Transfac 4.0 (http://transfac.gbf.de/TRANSFAC), several potential transcription factor binding sites (100% core similarity) were identified in the putative promoter region of *CDC2L1* gene. Among them, CAAT,[20] TCF11[21] and MZF1[22] were of most interest. CAAT is eukaryotic RNA polymerase II promoter elements. The C-48T and G-53C polymorphisms are located just beside its binding site. TCF11 is a widely expressed human transcription factor. It can interact with small Maf proteins and bind to a subclass of AP1 sites. The T-103C polymorphism is located within the core-binding site of TCF11. MZF1 is myeloid zinc finger gene 1, a transcriptional regulator of hematopoiesis. The T-210C polymorphism is located within the core-binding site of this transcription factor. The binding sites of these transcription factors are located besides or within the 4 polymorphism variants, suggesting that these variants might play a role in regulation of *CDC2L1* expression.

In addition to the 4 polymorphisms, we also noted sequence difference in the 5′ putative promoter region compared with the published *CDC2L1* sequence (GenBank accession number AF080684). Specifically, they were at −73 (G rather than C), at −49 (T rather than G), at 82 and 83 (GC rather than CG) and between 32 and 33 (1 more C). In addition, an A was present in the GenBank sequence at −80 but was not observed in any of the samples sequenced in our study.

Determination of methylation status of the putative CpG island

Our previous work[11] has shown that several melanoma cell lines had decreased levels of PITSLRE expression compared with normal melanocytes. Since we did not observe a high frequency of mutation and the promoter region of the *CDC2L1* gene contains a CpG island, we were interested in determining the methylation status of the 5′ promoter region of *CDC2L1* gene. To determine the presence of 5-methylcytosine in the 940 bp sequence of the *CDC2L1* 5′ region containing putative CpG island,[22] bisulfite modification PCR was carried out. The nested PCR amplification extended from −428 to +514. (The first nucleotide of exon 1 of *CDC2L1* is numbered +1.) Sodium bisulfite genomic sequencing was used to assess the cytosine methylation status of CpG dinucle-

T-103C and T-210C appeared identical in all samples examined, suggesting that the 2 changes could be in complete linkage disequilibrium. For melanoma cell lines, similarly, change G-53C was found to be a rarer polymorphism.

To confirm the nucleotide variants obtained by DNA sequencing, the existence of polymorphisms in the putative promoter region was also tested by restriction digestion. A representative PCR restriction length analysis of change 1 is shown in Figure 2. The restriction enzyme *Xho*I was found to cleave the 309 bp

otides of the *CDC2L1* 5′ region in human normal melanocytes, fibroblasts, melanoma cell line A375 (expressing normal levels of PITSLRE) and melanoma cell lines UACC 502, UACC 827, UACC 1227 and UACC 1237 (expressing low levels of PITSLRE).[11]

The sequence information obtained for each cell line was compared with the known sequence of the *CDC2L1* gene. Direct DNA sequencing showed that all CpGs in the putative CpG island were completely unmethylated in normal controls, normal PITSLRE-expressing melanoma cell line A375 and 5 low-PITSLRE-expressing melanoma cell lines (data not shown). These results indicated that, unlike some tumor suppressor genes that contain CpG island promoters in which aberrant methylation is associated with gene repression, the transcriptional repression of the *CDC2L1* gene was not associated with aberrant cytosine methylation in the putative *CDC2L1* CpG island.

DISCUSSION

To determine whether genetic alterations occur in the *CDC2L1* gene, we analyzed the full coding region (exons 2–19), 5′ UTR (exon 1 and part of exon 2) and the putative promoter region of the gene in 20 melanoma cell lines and 11 melanoma family samples. Thorough investigation of the coding region of *CDC2L1* revealed only 1 mutation at exon 7 in a melanoma cell line (UACC 903) and therefore led us to search for other alterations in noncoding regions, which might possibly be responsible for predisposition to melanoma as well.

Recently, attention has increasingly turned to investigation of the control sequences of tumor suppressor genes. Sequence changes in a promoter have been shown in a number of cases to reduce the level of transcription of some genes, resulting in familial diseases.[23,24] A single nucleotide polymorphism in the 5′UTR, which created an aberrant initiation site of the *CDKN2A* gene, has recently been identified in a subset of Canadian melanoma families; it was concluded that this polymorphism was associated with a predisposition to melanoma.[23] About 1 kb of the *CDC2L1* sequence around exon 1 (nt −527 to nt 559) was analyzed in an attempt to identify causal mutations in melanoma cell lines and melanoma families. PCR and sequence analysis revealed 4 different variants in the *CDC2L1* promoter, at −48, −53, −108 and −210 (Table II). All these variants detected in the putative *CDC2L1* promoter were shown to be present in the control population, indicating that they are probably polymorphisms.

Although these variants were demonstrated to be present in the panel of control DNA, the possibility of segregation of these variants with disease could not be ruled out. Howell *et al.*[25] reported recently that interleukin-10 (IL-10) promoter polymorphisms influence tumor development in cutaneous malignant melanoma (CMM). Genotypes associated with high levels of IL-10 expression *in vitro* are protective in CMM, whereas low expression genotypes are a risk factor for more advanced/poorer prognosis disease and may confer susceptibility to CMM. In addition, invasiveness of CMM was found to be influenced by matrix metalloproteinase 1 gene promoter polymorphism.[26] Therefore, further work on the effect of these variants on PITSLRE protein expression and their segregation with disease in melanoma families may be warranted.

There are several previous reports of promoter mutations that reduce the level of gene transcription. Those that have been reported tend to disrupt important regulatory regions in the promoter, such as the Sp1 binding site (or GC box),[24] and the TATA box.[27] In addition, Guo *et al.*[28] reported that a single nucleotide polymorphism between 2 closely spaced Myc-binding elements (E-boxes) in the promoter/regulatory region of *ODC* (the ornithine decarboxylase gene) affected the expression of this gene and that individuals homozygous for the A allele are capable of greater *ODC* expression after environmental exposures. A search for the consensus sequences of cis-acting promoter elements in the putative *CDC2L1*

promoter sequence revealed a possible location for CAAT box at nt −49 to nt −52. Changes 1 (T-48C) and 2 (G-53C) were found to locate exactly at 3′ and 5′ of the CAAT box. For change 1, 6 of 20 cell lines exhibited a genotype different from that of normal melanocytes, but only 1 of 20 cell lines exhibited a different genotype at change 2. Therefore, it is possible that the single-nucleotide polymorphisms C-48T and G-53C (especially C-48T) in the promoter region of *CDC2L1* could have functional significance. The variants T-103C and T-210C were found to create DNA binding sites for the transcriptional factors TCF11 and MZF1, respectively. The influence of each genotype (A1/A1, A1/A2 and A2/A2) on *CDC2L1* expression is under study.

In addition to the alterations of nucleotides in the promoter region, methylation of CpG islands in the promoter region of tumor suppressor genes has been shown to be an important mechanism of transcriptional repression/silencing and may play role in tumorigenesis.[29,30] The presence of methylated CpG islands in the promoter region of genes can suppress their expression. This process may be due to the presence of 5-methylcytosine, which apparently interferes with the binding of transcription factors or other DNA-binding proteins to block transcription. In different types of tumors, aberrant or accidental methylation of CpG islands in the promoter region has been observed in many cancer-related genes, resulting in the silencing of their expression.

For example, *p16^{INK4a}* promoter methylation was reported to be a common event in uveal melanoma and was accompanied by the loss of *p16^{INK4a}* expression.[31] For the *CDC2L1* gene, it has been reported that 2 distinct promoters, containing CpG-rich sequences corresponding to a previously isolated CpG genomic sequence characterized by Bird and colleagues, are responsible for the expression of transcripts from *CDC2L1* gene.[10] However, our study on the methylation status of melanoma cell lines showed that there was no aberrant methylation of CpG islands in the *CDC2L1* promoter and thus the decreased PITSLRE expression in these melanoma cell lines was not caused by hypermethylation of the CpG island.

The present study is consistent with the findings of Poetsch *et al.*,[32] who also found a low frequency of mutation in the *CDC2L1* gene in archival malignant melanoma specimens. However, we did not find any of the polymorphisms described by Poetsch *et al.*,[32] possibly because we compared potential base changes to the known sequences of both the *CDC2L1* and *CDC2L12* genes. Collectively, the results from both studies suggest that mutations in *CDC2L1* occur at low frequency in melanoma. It is also worth noting that several genes located at 1p36, such as p73,[33,34] PLA2G2A[35] and RPA[36] have been investigated in an attempt to identify a 1p36 tumor suppressor gene. Like *CDC2L1*, gene alterations within the coding region of these genes appear to be infrequent or nonexistent. This finding suggests that inactivation of tumor suppressor genes on 1p36 may not occur through the classical Knudson "2-hit" hypothesis.[37] We propose that haplo insufficiency of *CDC2L1* gene and its protein product, PITSLRE protein kinase, might be more important than somatic mutation as a mechanism of gene inactivation. This hypothesis is supported by recent spectral karyotyping findings in melanoma cell lines that indicate part or complete loss of 1p and cytogenetic data that demonstrate frequent abnormalities of 1p in melanoma.[38,39] However, further studies are necessary to clarify this issue. The same may be true for other potential tumor suppressor genes located at 1p36. Alternatively, the decreased protein expression seen in our previous studies could be due to the polymorphic changes observed in the present study or impaired translation processing of *CDC2L1* mRNA transcripts.[40]

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